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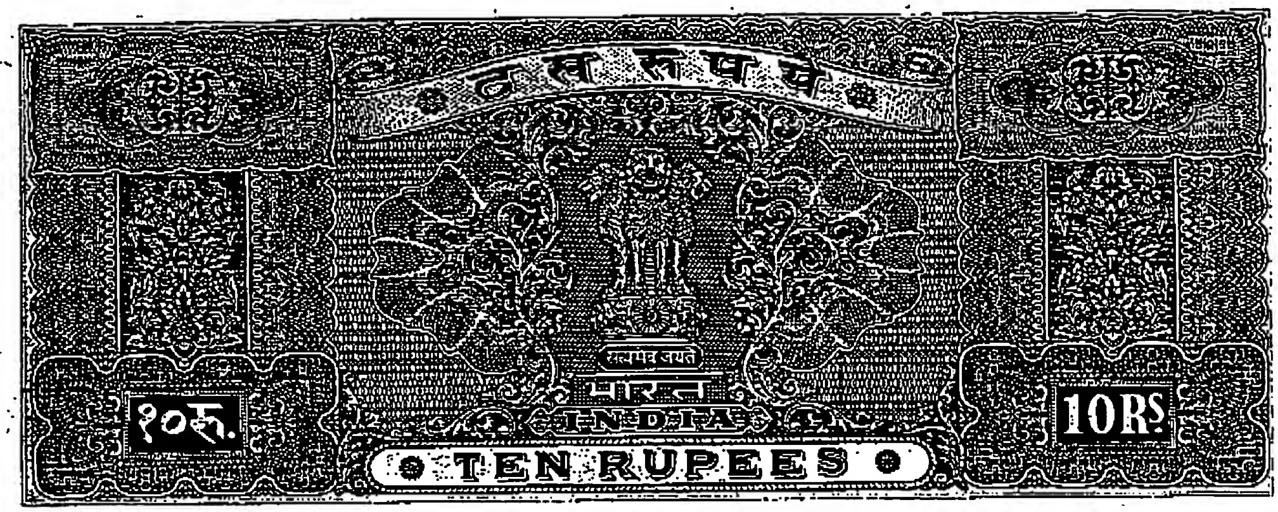

S/N 09/867,367 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Kumar et al.	Examiner:	S. Wang
Serial No.	09/867,367	Group Art Unit	1617
Filed:	May 29, 2001	Docket No. :	11378.14USCI
Title:	A MICROBIAL COMPOSITION AND A PROCESS FOR THE NEUTRALIZATION OF ALKALINE WASTE WATERS		

Declaration under 37 C.F.R. 1.132 by Rita Kumar

I, Rita Kumar, age 51 years, residing at C 6B/79 Janak Puri, New Delhi, India, citizen of India do hereby state as under:

1) I am a Scientist at CBT, Mall Road, New Delhi, India. I graduated in 1971 from Jammu and Kashmir University located at Srinagar, India. I completed my Master's Degree from Haryana Agricultural University at Hissar, India in 1973-74. Subsequently, I graduated with a doctoral degree from Delhi University in 1982-83.

2) After completing my doctoral degree, I took my first position as a Scientist with CBT (1976 till 1980). Subsequently, I took my first position as a Scientist with CBT in 1982. I was employed at CBT for 26 years. I have been a Senior Scientist with CBT since 1988.

Thus, I have been working in the field of microbial technology for the last 26 years.

1976-1980	JSA	Quality control (testing of antigens)
1980- 1982	SSA	Quality control (testing of antigens)
1982- 1983	Scientist A	Quality control (testing of antigens)
1983- 1988	Scientist B	Quality control (testing of antigens) and research project on Hypersensitivity Pneumonitis

1988-1993	Scientist C	Quality control (testing of antigens) and research projects on Environmental biotechnology
1993-1998	Scientist EI	Quality control (testing of antigens) and research projects on Environmental biotechnology
1998	-	Quality control (testing of antigens) and research projects on Environmental biotechnology

3. The projects I am currently a part of include:

   A. Technological development of specific microbial packages for treatment of industrial waste-waters. *Sponsored* - Department of biotechnology, Started-2000, Duration – 3 Years.

   B. Treatment of industrial effluent by a novel bio-physico technology involving specific microbial packages and innovative flotation Techniques. *Sponsored* – Department of Biotechnology, Started-2000, Duration – 3 Years.

   C. Extensive testing of the developed mixed culture based BOD biosensor for determining the pollutional load of industrial waste-waters – a device for rapid non-conventional monitoring – *Sponsored* Ministry of Environment & forests, Started – 2002, Duration – 3 Years

4. The projects I have investigated in the past include:

   A. *Rate of Thermophilic Actinomycetes in Extrinsic allergic Alveolitis (EAA) / Hypersensitivity Pneumonitis (HP).* Sponsored – In-house, 1992

   B. A Microbial composition and a process for the neutralization of alkaline waste waters" In-house 1992.

C. *Development of Suitable Antigens for the Diagnosis of Hypersensitivity Pneumonitis* Sponsored – Department of Science & Technology, 1993-98.

D. *Standardization of Reference Seed Culture for the BOD estimation. Sponsored* - Ministry of Environment & Forests, 1994-97.

E. *Testing of Microbial Mixed Seed Culture in BOD Determination – Sponsored* – Central Pollution Control Board, Delhi, 1994-95

F. *Testing of Pharmaceutical* Samples for its Antimicrobial Potency study Consultancy Themis Chemicals Ltd. Bombay, 1994.

G. *Testing of Disinfectant for its Antimicrobial Effectiveness and its comparison with Povidone-iodine. Consultancy – Unichem Laboratories Ltd.. Bombay. 1995.*

H. *Antimicrobial Analysis of Fugocide* Consultancy Themis Chemicals Ltd., Bombay, 1996-97.

I. Development of Mixed Culture Based BOD Biosensor for Instant and Reproducible Monitoring of BOD Load in Waste – Waters. *Sponsored* – Ministry of Environment & Forests, Started 1998-2001.

5. One of the projects undertaken by CBT is "A Microbial composition and a process for the neutralization of alkaline waste waters". This project was begun in 1992. The scientists involved in the study were Rita Kumar (myself), Anil Kumar, Alka Sharma, Sharad V. gangal and Santosh D. Makhijani. I was one of the main scientists in this study. I am aware of US patent application No. 09/867,367 filed on the subject matter of this project. I am also aware and familiar with all the office actions, objections of the Examiner, and the references cited by the Examiner. Therefore, I am completely and fully aware of all the facts relating to this project as well as the present patent application.

6. A general discussion of the invention:

a) Background

Waste water generated from industries is a major cause of surface water pollution. Some of the waste water from industrial processes pose a major problem for treatment because they are alkaline in nature. Industries

producing alkaline waste water include textile industries, paper and pulp industries and others. This waste water has pH ranging from 8.0 - 11.0. Since the pH of the waste waters is greater than 9, it is not permissible to drain these waste-waters without treatment.

One common solution is to neutralize the waste waters prior to releasing them. One method of neutralizing the waste water is to treat it with acids. However, use of large quantities of acids is unsafe and likely to pose health hazards for workers.

A better alternative would be to treat the waste waters biologically to neutralize them.

b) Solution

The present invention is directed toward this end. We have developed a composition comprising two bacteria that have been adapted to grow in highly alkaline waste water environments and neutralize such waters.

The bacteria selected for the microbial composition are *Bacillus alkalophilus* and *Bacillus sp.* having characteristics similar to the organism deposited at ATCC No.27647 and ATCC No.27557 respectively. The characteristics of the bacteria are set out at pages 6 and 7 of the specification. The bacteria have been isolated from waste water effluents of textile industries. These bacterial strains labelled as CBTCC/Micro/8 and CBTCC/Micro/9 have been deposited at the Microbial Culture Collection at Chandigarh, India, an International depository, recognized under the Budapest Treaty.

Therefore, the invention provides a process for the preparation of a microbial composition consisting of two alkalophilic bacteria isolated from waste effluents.

The environments in which the bacteria are found are slightly alkaline waters, and therefore, after testing, we found that these bacteria are incapable of living in waters having a pH above 9.0 at the time of isolation.

The alkalophilic bacterial strains have been isolated by us from the sewage waters which have an alkaline pH of 7.5 to 8.5. The peculiarity of these bacteria is that each of the bacteria are capable of growing at a high pH range i.e. 8.0 to 11.0, but not at a neutral pH. If these bacteria are grown in an alkaline environment phenotypic alterations are found during the exponential growth phase (see page 3 and 4 of the specification). Therefore, these bacteria are adapted to survive only up to a certain pH in alkaline waste waters. Beyond that pH, these bacteria cannot survive.

If the alkalophilic strains are used as such to neutralize alkaline waste waters, the strains will neither grow nor neutralize (**see table 9a to 9d attached herewith**).

Hence, they cannot be directly used to neutralize waste waters. We therefore thought it necessary to acclimatize the bacteria to an alkaline environment.

Acclimatization is the process of adaptation to environmental change, wherein a population of a specific strain of bacteria increase over successive generations in response to its environment. In the case of bacteria that undergo acclimatization, the bacteria adapt to a new environment. Often, the modifications include enzymatic changes enabling the bacteria to utilize nutrient sources for survival.

An important aspect of acclimatization is that it is unique for each bacterium. There is no standard process that can be blindly used for all bacteria. In fact, the process used for one bacterium may not work for another. The bacteria may not respond to the process or may not become acclimatized. While a skilled person is generally aware that a process for acclimatization exists, each bacterium involves certain unique techniques.

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For the purposes of the invention, the bacterial strains CBTCC/Micro/8 and CBTCC/Micro/9 were grown individually and were acclimatized to an alkaline pH. The acclimatization of the bacterial strains was carried with the following procedures:

a) the isolated bacterial cultures of *Bacillus* species and *Bacillus alkalophilus* were individually subcultured in Tryptose Soya Broth (TSB) at pH values increasing up to pH 11.0.
b) the cultures were grown in a medium of a higher pH and several sub-cultures were made,
c) after the confluent growth, the bacterial cultures were sub cultured to a medium of higher pH.

The bacteria were grown under normal conditions as described. The medium consisted of Tryptose Soya Broth (TSB) at pH values in increasing order. The medium was changed after confluent growth was observed. The population of bacteria was gradually increased and a number of subcultures of bacterial strains of CBTCC/Micro/8 and CBTCC/Micro/9 were done according to the above process. After considerable confluent growth, the bacterial strains of CBTCC/Micro/8 and CBTCC/Micro/9 were isolated and used in other steps of the invention.

Both the modified strains of Bacillus sps and *Bacillus alkalophilus* i.e bacterial strains CBTCC/Micro/8 and CBTCC/Micro/9 were used individually to neutralize the alkaline waste-waters (having pH 11.0) to desired level i.e., neutral pH range (pH 7.0-7.5).

In order to achieve neutralization of waste-water to a desired level, both the bacterial strains were used individually to neutralize the waste waters.

The results of the observation are given below:

Both the modified strains of *Bacillus sps* and *Bacillus alkalophilus* were used individually to neutralize the alkaline waste water (having pH 11.0) to the desired level i.e., neutral pH range (pH 7.0-7.5). When used individually, these strains could bring down the pH of alkaline waste water (textile) only to a certain level i.e.,0.24 to 1.06 pH units and 0.25 to 1.03 pH units by Bacillus alkalophilus and Bacilluss species respectively. It is clear from Tables 6 and 7 that the bacteria CBTCC/Micro/8 and CBTCC/Micro/9 individually even upon acclimatization,are unable to neutralize the waste waters to a satisfactory level.

It is clear from these Tables that even upon acclimatization, CBTCC/Micro/8 and CBTCC/Micro/9 are unable to neutralize the waste waters to a satisfactory level.

This made us change the process. We decided to add some carbon source to the waste waters so that the bacteia can utilize it and neutralize the waste waters.

A further experiment was conducted where we tried the neutralization of the waste waters by individual strains of bacteria in the presence of a carbon or sugar source.

We found that when both bacterial strains CBTCC/Micro/8 and CBTCC/Micro/9, were used individually in the presence of carbohydrates, i.e., 1% sucrose and 1% glucose, the change in pH units was in the range of 1.09 to 1.77 and 1.22 to 1.55 by *Bacillus alkalophilus* respectively (Table 9b of the patent application).

On the other hand, *Bacillus* sp was able to bring a change in pH units in the range of 1.11 to 1.89 in the presence of 1% sucrose and 1.30 to 1.94 in the presence of 1% glucose as shown in table 6 of the patent application (Table 9a of the patent application).

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In case f textile effluents, the pH of the effluent is quite high i.e., 11.0, and if the reduction is only 1-2 pH units as observed using said individual bacterial strains, it will not serve the purpose of neutralization of the high pH waste-waters.

The desired result was not obtained and this made us try another alternative. We decided to use the strains in combination and in the presence of a carbon or sugar source for the neutralization of waste-waters.

As a combination, a pellet of the strains CBTCC/Micro/8 and CBTCC/Micro/9 was prepared and tested. The combination showed a degree of synergism, by changing the pH 0.45 to 1.13 pH, which is still noticeably higher as compared to the performance by individual bacterial strains CBTCC/Micro/8 and CBTCC/Micro/9. The results are depicted in Table S.

Thus, the skill of the applicant was tested in

- Proposing a novel composition that is capable of neutralizing alkaline waste waters
- The finding that the bacteria cannot be simply used for neutralizing alkaline waters. These bacteria need to be acclimatized to high pH before they can be put to such use
- Finding the specific pH range at which the bacteria need to be acclimatized.

7. Why the invention is unexpected in light of the prior art:

The Examiner has cited several references that are asserted to render the invention obvious. I have reviewed these references in the light of the objections raised by the Examiner.

My comments are as follows:

a) Boyer et al International Journal of Systemic Bacteriology 1973, pg 238-242 "U." : this publication is a general study of alkaline-amylase

producing bacteria. The bacterial strain NCTC 4553 is compared with two other bacterial strains NRRL B-3881 and ATCC 21591. The strain NCTC 4553 is isolated from human faeces. (see col 1). The study describes some of the properties of this bacterium.

b) Boyer et al - Journal of baceriology, 1972 pg 992-1000"V": This publication reports the production of an unusual alkaline amylase from Bacillus species. The *Bacillus* strain B-3881 reported is said to be active at a pH of 10 to 11.

c) US 4511657: This patent contemplates the use of activated sludges that have been acclimatized to metabolize the recalcitrant obnoxious organics. The patent refers to acclimatization. It may be noted here that acclimatization as a general scheme is known to a person skilled in the art. However, the invention lies in developing a separate process for acclimatization of the alkalophilic bacteria as described earlier.

d) Horikoshi et al (US 4052262) describes the production of an alkaline protease by a strain of the Bacillus genus. The protease produced is claimed to be a useful additive for detergents. The medium used for culturing the bacteria has a high carbonate content and is supplemented with salts.

e) Takowa et al. teach that alkaline waste water can be treated by using any bacterium selected from alkaline bacteria and *bacillus sp.* These bacteria are different from those used in the present invention. Moreover, Takowa et al do not teach that when the bacteria are directly used for treatment of alkaline waste water, problems are encountered in the growth phase of the bacteria and these bacteria should be acclimatized to alkaline environment. Also, Takowa does not teach microbial compositions containing both *Bacillus sp.* as well as alkalophilic bacteria.

f) Jones: This publication teaches that bacteria ar cultivated in an alkaline environment before harvesting them.

According to the Examiner, a skilled person would find it obvious to select an alkalophilic bacteria, acclimatize it to suit waste water treatment through the production of acids. The references like Takowa teach that Bacillus sp. can be used for treating alkaline waste waters.

I, as a person skilled in the art would consider the invention at hand a process for the preparation of a composition consisting of an equal amount of bacterial strains unexpected in light of the cited prior art.

The problem with the alkalophilic bacteria which the invention utilizes is that initially they cannot survive in a high pH environment. Therefore, they require acclimatization. These bacteria have been isolated from slightly alkaline medium i.e. pH 7.5 to 8.0 and acclimatized to a higher pH i.e. 11 pH.

However, the Examiner incorrectly assumes that upon acclimatization, the acclimatized bacterium will automatically produce acids and neutralize the waste waters.

This assumption is not true. It is shown by the fact that the bacteria, when grown individually in alkaline waste water, could not neutralize the waste water. At a pH of 8 to 8.5, the activity was nearly nil. The results of this study in terms of values recorded after 2 days, after 4 days and after 7 days are shown in Tables 9(A to D).

Only if both the micro-organisms, i.e. the acclimatized CBTCC/Micro/8 and CBTCC/Micro/9 are used as a combination, in the presence of a carbon or sugar source, will the desired results, i.e. reducing the pH value of the alkaline waste-water of textile industry from 11.0 to 7.0-7.5, be achieved.

There is nothing in the prior art that suggests that:

- If the bacteria CBTCC/Micro/8 and CBTCC/Micro/9 do not neutralize the waste waters, a combination should be prepared. Furthermore, the ratio in which they should be combined is also not taught by the prior art

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- If the combination is not satisfactory, then a carbon or sugar source should be added. It is also not taught how much of this source should be added.

The prior art only teach media for culturing various bacteria. This does not teach that addition of a carbon or sugar source to the medium enables the bacteria to neutralize waste waters.

Once the bacteria have been acclimatized to a highly alkaline environment, they can then neutralize waste waters. However, as can be seen from Tables (9a-b), this does not happen. It is further unexpected that

- these specific bacteria can neutralize waste waters
- they need to be acclimatized before they can be introduced into waste water environment
- after acclimatization, a combination of bacteria needs to be prepared
- a carbon or sugar source must be added to the waste waters to enable the bacteria to neutralise waster waters.

The cited art does not teach or suggest any of the above principles. Specifically, the cited art does not teach that neutralization studies can be conducted using the bacteria individually and in combination, in the presence of carbohydrates.

8. **Summary of Unexpected Findings**

Both the modified strains of *Bacillus sp.* and *Bacillus alkalophilus* were used individually to neutralize the alkaline waste-water (having pH 11.0) to a desired level, i.e., neutral pH range (pH 7.0 - 7.5). These strains when used individually, could bring down the pH of alkaline waste-water (Textile) to only a certain level i.e., 0.24 to 1.06 pH units and 0.25 to 1.03 pH units by

Bacillus alkalophilus and Bacillus species respectively. This is represented in Tables 6 & 7.

When both the acclimatized strains CBTCC/Micro/8 and CBTCC/Micro/9 are used in combination for neutralization of textile waste-waters, they showed a degree of synergism, as is evident by the change in pH units from 0.45 - 1.13, which is noticeably higher as compared to the individual bacterial strains. This is indicated in Table 8 of the patent application.

When the acclimatized strains CBTCC/Micro/8 and CBTCC/Micro/9 are grown in the presence of carbohydrates (i.e. 1% sucrose and 1% glucose), the neutralizing capacity of the bacteria is increased.

When the acclimatized strain Bacillus alkalophilus is grown in the presence of 1% sucrose and 1% glucose, a reduction in pH from 1.09 to 1.77 and 1.22 to 1.55 respectively is observed. This is shown in table 9b of the patent application.

When the acclimatized strain Bacillus sp. is grown in the presence of 1% sucrose, it was able to bring change in the pH units in the range of 1.11 to 1.89 and, when it is grown in 1% glucose, a reduction in the pH from 1.30 to 1.94 is observed (see table 9a of the application).

When both the acclimatized strains are grown in the presence of carbohydrates, a change of approximately 4 pH units is observed, bringing the pH down to a neutral range, i.e. 7.0 - 7.5. This is shown in Table 8 of the patent application. This indicates that Bacillus alkalophilus, Bacillus sp. act in a synergistic fashion in the presence of carbohydrates

Therefore, it is only a combination of these bacterial strains CBTCC/Micro/8 and CBTCC/Micro/9 that is useful in neutralising alkaline waste waters.

None of the cited prior art teach or suggest methods or solutions to treat alkaline waste waters from textile industries. The pri r art also does not

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teach that alkalophilic bacteria in combination with bacillus sp. can be used to develop a composition which can be used to neutralise alkaline waste water.

9. **Conclusion:**

None of the citations referred to by the Examiner teach or suggest a process for the composition of the present invention. Therefore, the invention that is the subject matter of this patent application is unexpected in view of the prior art.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Dated: 21/03/2003

Rita Kumar

Table 9(a)

Neutralization of alkaline waste-water from textile industry by *Bacillus* sps in the presence of carbohydrates

Carbohydrate source	Original pH Of waste-water	After 4 days of incubation		After 7 days of incubation	
		pH	Change in pH unit	pH	Change in pH units
With 1% Sucrose	10.67	9.04	1.63	8.78	1.89
	11.57	9.98	1.59	9.84	1.73
	9.88	8.53	1.35	8.40	1.48
	12.01	10.90	1.11	10.74	1.27
	9.65	8.45	1.20	8.33	1.32
With 1% Glucose	10.67	8.94	1.73	8.73	1.94
	11.57	9.97	1.60	9.74	1.83
	9.88	8.47	1.41	8.34	1.54
	12.01	10.57	1.44	10.41	1.60
	9.65	8.35	1.30	8.18	1.47

Table 9 (b)

Neutralization of alkaline waste-water from textile industry by *Bacillus alkalophilus* in the presence of carbohydrates

Carbohydrate source	Original pH Of waste-Water	After 4 days of incubation		After 7 days of incubation	
		pH	Change in pH units	pH	Change in pH units
With 1% Sucrose	10.67	9.09	1.58	8.90	1.77
	11.57	10.17	1.40	9.96	1.61
	9.88	8.57	1.31	8.45	1.43
	12.01	10.92	1.09	10.80	1.21
	9.65	8.52	1.13	8.36	1.29
With 1% Glucose	10.67	9.06	1.61	8.82	1.85
	11.57	10.05	1.52	9.86	1.71
	9.88	8.48	1.40	8.35	1.53
	12.01	10.60	1.41	10.44	1.57
	9.65	8.43	1.22	8.29	1.36

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Table 9 (c)

Neutralization of alkaline waste-water from textile industry by *Bacillus* sp. Growing at original pH i.e., 8.0 & 8.5.

Source	Original pH Of waste-water	After 4 days of incubation		After 7 days of incubation	
		pH	Change in pH unit	pH	Change in pH units
Without carbohydrates	10.67	16.67	Nil	10.67	Nil
	11.57	11.57	Nil	11.57	Nil
	9.88	9.88	Nil	9.88	Nil
	12.01	12.01	Nil	12.01	Nil
	9.65	9.65	Nil	9.65	Nil
With 1% Sucrose	10.67	10.65	0.02	10.62	0.05
	11.57	11.56	0.01	11.53	0.04
	9.88	9.87	0.01	9.85	0.03
	12.01	12.01	0.00	11.97	0.04
	9.65	9.64	0.01	9.60	0.05
With 1% glucose	10.67	10.63	0.04	10.60	0.07
	11.57	11.54	0.03	11.52	0.05
	9.88	9.85	0.03	9.83	0.05
	12.01	11.97	0.04	11.95	0.06
	9.65	9.63	0.02	9.61	0.04

Table 9 (d)

Neutralization of alkaline waste-water from textile industry by *Bacillus alkalophilus* growing at original pH i.e., 8.0 and 8.5

Source	Original pH Of waste-water	After 4 days of incubation		After 7 days of incubation	
		pH	Change in pH unit	pH	Change in pH units
Without carbohydrates	10.67	10.67	Nil	10.67	Nil
	11.57	11.57	Nil	11.57	Nil
	9.88	9.88	Nil	9.88	Nil
	12.01	12.01	Nil	12.01	Nil
	9.65	9.65	Nil	9.65	Nil
With 1% Sucrose	10.67	10.64	0.03	10.63	0.04
	11.57	11.55	0.02	11.53	0.04
	9.88	9.87	0.01	9.83	0.05
	12.01	12.01	0.00	11.98	0.03
	9.65	9.63	0.02	9.61	0.04
With 1% glucose	10.67	10.64	0.03	10.61	0.06
	11.57	11.53	0.04	11.51	0.06
	9.88	9.85	0.03	9.83	0.05
	12.01	11.97	0.04	11.94	0.07
	9.65	9.62	0.03	9.60	0.05